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Cellular Compartmentation and the Redox/Nonredox Functions of NAD^+

Chaitanya A. Kulkarni and Paul S. Brookes

Abstract

Significance: Nicotinamide adenine dinucleotide (NAD^+) spans diverse roles in biology, serving as both an important redox cofactor in metabolism and a substrate for signaling enzymes that regulate protein post-translational modifications (PTMs).
Critical Issues: Although the interactions between these different roles of NAD^+ (and its reduced form NADH) have been considered, little attention has been paid to the role of compartmentation in these processes. Specifically, the role of NAD^+ in metabolism is compartment specific (*e.g.*, mitochondrial *vs.* cytosolic), affording a very different redox landscape for PTM-modulating enzymes such as sirtuins and poly(ADP-ribose) polymerases in different cell compartments. In addition, the orders of magnitude differences in expression levels between NAD^+-dependent enzymes are often not considered when assuming the effects of bulk changes in NAD^+ levels on their relative activities.
Recent Advances: In this review, we discuss the metabolic, nonmetabolic, redox, and enzyme substrate roles of cellular NAD^+, and the recent discoveries regarding the interplay between these roles in different cell compartments.
Future Directions: Therapeutic implications for the compartmentation and manipulation of NAD^+ biology are discussed. *Antioxid. Redox Signal.* 31, 623–642.

Keywords: nicotinamide adenine dinucleotide, redox, mitochondria, metabolism, glycolysis, compartmentation

Introduction

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) functions both as a redox cofactor in metabolism and as a substrate for several families of enzymes that regulate protein post-translational modifications (PTMs), including the silent information regulator 2p homolog family of lysine deacylases (sirtuins, SIRTs) and the poly(ADP-ribose) polymerases (PARPs). Given that NAD^+ availability *in vivo* is likely to be limited, the general concept of competition for NAD^+ between these different roles (redox cofactor *vs.* substrate) has not been extensively explored. Herein, we address the role of compartmentation in regulating the balance between NAD^+ redox *versus* substrate roles. We focus on the cytosolic, mitochondrial, and nuclear compartments, since these are the most studied, with NAD(H) in other compartments such as endoplasmic reticulum (ER) or peroxisomes being beyond the scope of this review [see (166) for review].

Although the redox couple between NAD^+ and its reduced form NADH is often considered alongside the redox couple between phosphorylated NAD^+ (*i.e.*, $NADP^+$) and its reduced form (NADPH), these reactions are normally exclusive—NAD(H)-dependent enzymes do not typically use NADP(H) and vice versa. While there are some exceptions [*e.g.*, glutamate dehydrogenase in mammals (39)], most enzymes (*e.g.*, isocitrate dehydrogenase [IDH]) exist as separate NAD(H)- or NADP(H)-dependent isoforms encoded by different genes. The focus of this review will be NAD^+, with $NADP^+$ only mentioned as it pertains to NAD^+ biology.

First, we discuss the synthesis and biological sources of NAD^+, followed by fates of NAD^+ both metabolic and redox, the interplay between these fates, and how compartmentation facilitates the different cellular roles of NAD^+. Throughout this review, therapeutic approaches to manipulate cellular levels of NAD^+ and its precursors or metabolites are discussed in the context of various diseases.

Department of Anesthesiology, University of Rochester Medical Center, Rochester, New York.

NAD^+ Synthesis and Compartmentation

Routes of NAD^+ biosynthesis

In mammalian cells, NAD^+ can by synthesized using various forms of vitamin B3—nicotinic acid (NA), nicotinamide (NAM), or nicotinamide riboside (NR)—as the starting point. Alternatively, NAD^+ can be synthesized *de novo* from tryptophan. NA, NAM, NR, and tryptophan are ingested through the diet and afford four main routes to NAD^+ (Fig. 1A–D). Intermediates of NAD^+ biosynthetic pathways such as nicotinamide mononucleotide (NMN) and alternative precursors such as nicotinic acid riboside (NAR) can also serve as sources for NAD^+ synthesis (Fig. 1E) (81, 135). Besides dietary sources, products of NAD^+-degrading enzymes such as SIRT, PARP, CD38, CD157 (all produce NAM), CD73 (NMN and NR), and NUDT13 (NMN) are also salvaged to replenish cellular NAD^+ pools (note: these enzymes are discussed, and abbreviations are defined in the NAD^+-Consuming Pathways and Compartmentalization section).

The three-step pathway for NAD^+ generation from NA is the Preiss–Handler pathway (Fig. 1A) (130, 131). The enzyme nicotinic acid phosphoribosyltransferase converts NA to nicotinic acid mononucleotide (NAMN) using the ribose-5-phosphate group of phosphoribosyl pyrophosphate (PRPP). NAMN is then adenylated by nicotinamide mononucleotide adenylyltransferase (NMNAT) using adenosine triphosphate (ATP) to yield nicotinic acid adenine dinucleotide (NAAD). Three isoforms of NMNAT have been reported—NMNAT1 is nuclear, NMNAT2 is in the cytosol and Golgi apparatus, and NMNAT3 is mitochondrial (12). In the final step, the cytosolic enzyme NAD^+ synthase amidates NAAD using L-glutamine and ATP to give NAD^+.

The two-step salvage pathway to recycle NAM into NAD^+ is similar to the first two steps of the Preiss–Handler pathway (Fig. 1B). Using PRPP, nicotinamide phosphoribosyltransferase (NAMPT) transforms NAM to NMN. This is the rate-limiting step in the salvage of NAM (136). NAMPT is primarily located in the nucleus and cytosol. Early research reported the presence of NAMPT in mitochondria (172), although more recently advanced techniques have led to this finding being disputed (33, 128). NMNAT adenylates NMN in the presence of ATP to give NAD^+. Since many NAD^+-consuming enzymes produce NAM as a product, this pathway is a key mechanism for the maintenance of cellular NAD^+ levels. Furthermore, NAM is a feedback inhibitor of NAD^+-consuming enzymes such as SIRTs, so swift removal of NAM is required to maintain these enzymes' activities.

Synthesis of NAD^+ from NR can proceed one of two ways (Fig. 1C). Predominantly, NR is phosphorylated by nicotinamide riboside kinases 1 and 2 (NRK1/NRK2) using ATP to give NMN (16), which is then converted to NAD^+ by the NMNATs. The phosphorylation step is rate limiting for the use of exogenous NR in NAD^+ synthesis (135). An alternate route involves the cleavage of the glycosidic linkage in NR by purine nucleoside phosphorylase (PNP) to make NAM, which subsequently enters the salvage pathway to give NAD^+ (10, 46). Besides NR, both NRKs and PNP also accept NAR as a substrate to give NAMN and NA as respective products (10, 158). Both compounds then feed into the Preiss–Handler pathway.

The *de novo* synthesis of NAD^+ from dietary tryptophan occurs *via* the kynurenine pathway (Fig. 1D) (11, 71). The first and rate-limiting step requires oxygen to convert L-tryptophan to *N*-formylkynurenine. This transformation is performed by two enzymes—indoleamine-2,3-dioxygenase and tryptophan-2,3-dioxygenase, the latter of which is liver-specific in mammals. Both these cytosolic enzymes are frequently overexpressed in a variety of cancers and confer immune resistance to cancer cells (68, 129, 177). The subsequent four steps include alternating hydrolysis and oxidation. Spontaneous hydrolytic removal of formate from *N*-formylkynurenine gives L-kynurenine, which is oxidized by kynurenine 3-monooxygenase to 3-hydroxy-L-kynurenine using oxygen and NADPH. This is followed by the formation of 3-hydroxyanthranilic acid (3-HAA) by removal of L-alanine. Subsequent oxidation of 3-HAA by 3-hydroxyanthranilic acid 3,4-dioxygenase gives an unstable intermediate aminocarboxymuconate semialdedhye (ACMS). ACMS can go two ways, only one of which yields NAD^+: Normally, enzymatic conversion of ACMS to 2-aminomuconate semialdehyde (AMS) by aminocarboxymuconate semialdehyde decarboxylase (ACMSD) occurs, with AMS subsequently converted to acetyl-CoA. Alternatively, when ACMSD is saturated by high concentrations of ACMS, it spontaneously cyclizes, forming quinolinic acid, which is then adenylated and decarboxylated by quinolinate phosphoribosyltransferase, another rate-limiting enzyme that gives NAMN, which feeds into the Preiss–Handler pathway (11).

Cellular uptake of NAD^+ precursors

Tryptophan is taken up into cells *via* carrier proteins that also transport large neutral amino acids (Fig. 2) (118). Among the different forms of vitamin B3, cellular uptake of NA is mediated by membrane carrier systems potentially including either a pH-dependent anion antiporter or a proton cotransporter (107, 144, 156). The exact mechanism of cellular NAM uptake, either as direct transport in intact form or conversion to salvage pathway metabolites, which are then taken up, is still unknown. NAMPT, the enzyme that converts NAM to NMN, is found both intracellularly and extracellularly, indicating that both routes of NAM uptake are possible. However, NAMPTs substrates ATP and PRPP were shown to be not available in sufficient quantities in the extracellular space (63). Studies in rodents have indicated that NAM is directly absorbed by the intestines (29, 55). The third form of vitamin B3, NR, is directly absorbed into cells with the help of equilibrative nucleoside transporters (ENTs) (Fig. 2) (111, 153).

NMN, an intermediate in the NAD^+ salvage pathway (Fig. 1B), can itself serve as an exogenous precursor for NAD^+ and has been shown to increase NAD^+ levels in mice (135). Until recently, there was debate regarding whether NMN is directly taken up by cells or must undergo metabolism before uptake. Several researchers proposed that NMN is extracellularly dephosphorylated to NR, which then enters the cell (Fig. 2) (9, 57, 111), whereas others suggested that the cell membrane is permeable to NMN (47). It has been argued that NMN dephosphorylation is likely a cell-type-specific phenomenon, and intact NMN can be rapidly transported into certain cell types such as hepatocytes (102, 176). However, recent studies showed that even in hepatocytes, NRK1 is necessary and rate limiting for the use of exogenous NMN for NAD^+ synthesis (135). This suggests that if NMN was

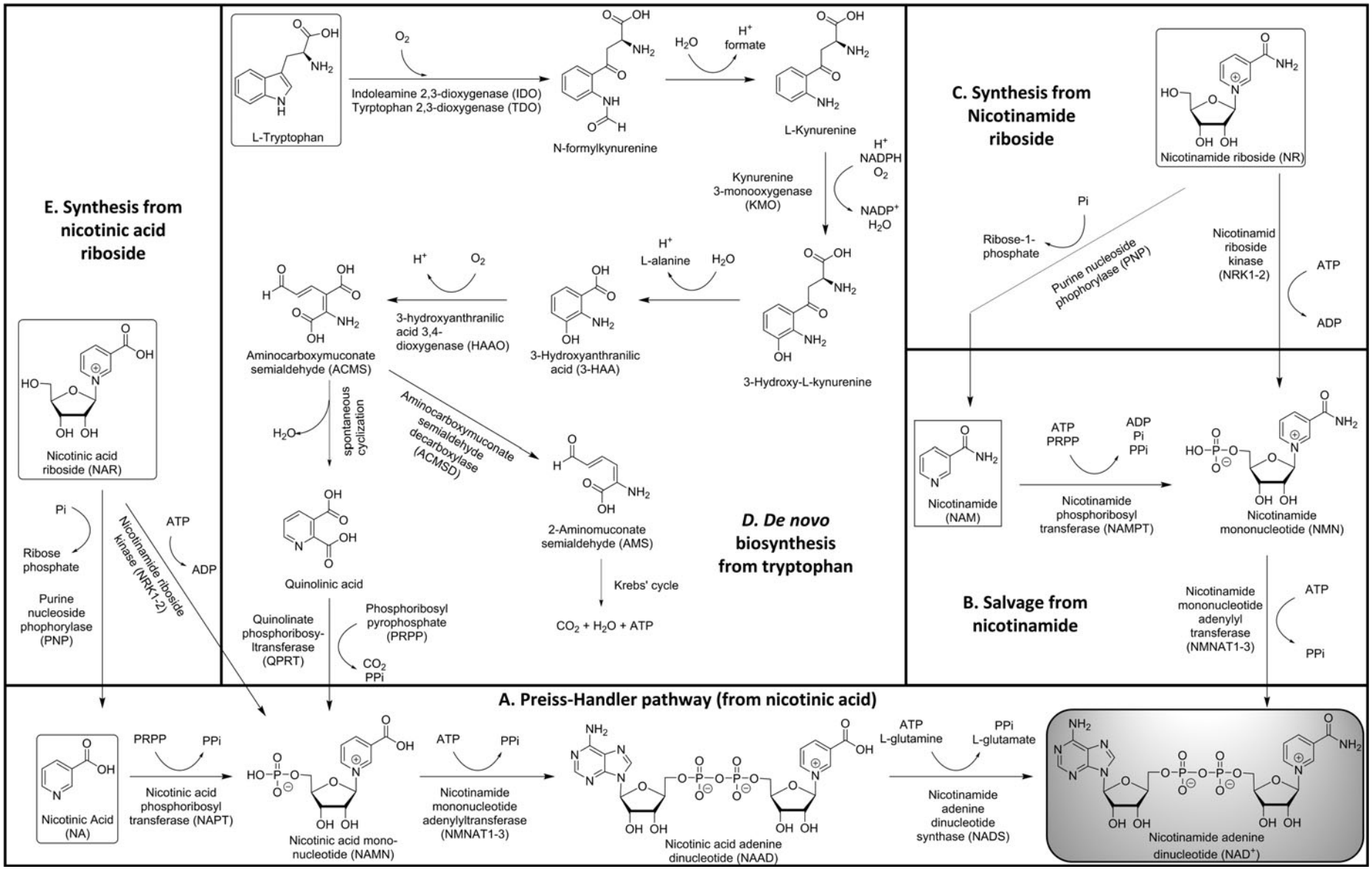

FIG. 1. Biosynthesis of NAD$^+$. Five biosynthetic routes to NAD$^+$ in mammalian cells are shown, with NAD$^+$ itself in the *gray box* at *lower right*. **(A)** The Preiss–Handler pathway is the main synthetic route to NAD from precursors such as NA. **(B)** NAD$^+$ can also be salvaged from NAM, the product of many NAD$^+$-consuming enzymes. **(C)** NMN can be synthesized from NR, which is orally bioavailable. **(D)** NAMN in the Preiss–Handler pathway **(A)** can be synthesized *de novo* from the amino acid tryptophan. **(E)** NA or NAMN can be synthesized from NAR. For full discussion, see text (the NAD$^+$ Synthesis and Compartmentation section). NA, nicotinic acid; NAD$^+$, nicotinamide adenine dinucleotide; NAM, nicotinamide; NAMN, nicotinic acid mononucleotide; NAR, nicotinic acid riboside; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside.

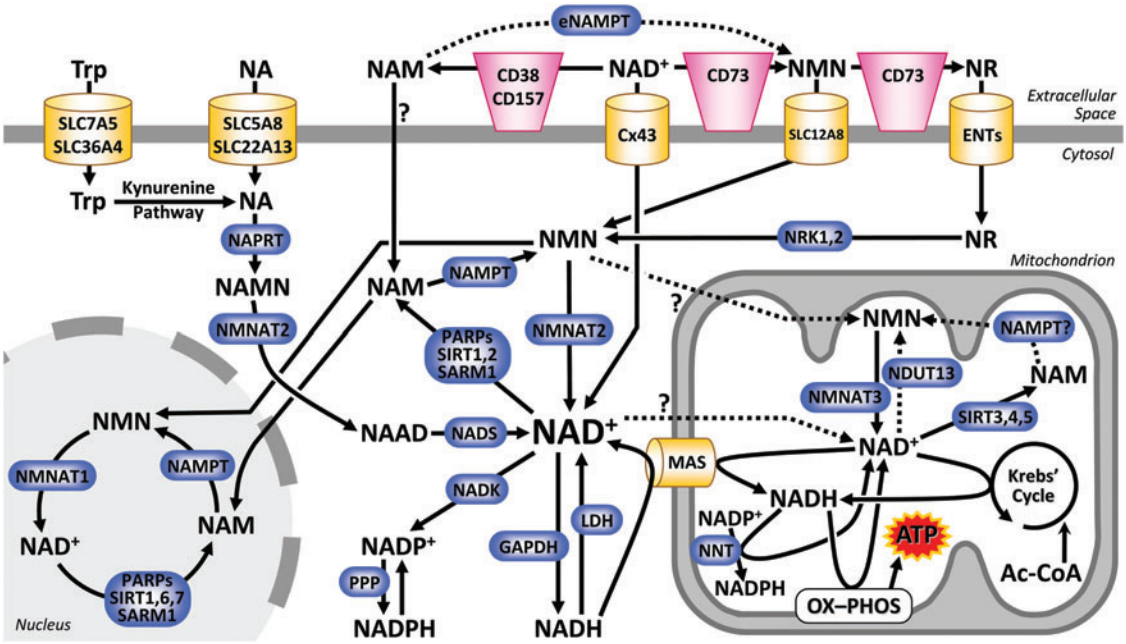

FIG. 2. Compartmentation of NAD$^+$ biology. The cellular compartments considered herein are cytosol, nucleus, mitochondrion, and extracellular space. The major routes for import, biosynthesis, and consumption of NAD$^+$ in each of these compartments are shown. *Dotted lines* and *question marks* denote pathways for which limited evidence is available. ATP, adenosine triphosphate; ENT, equilibrative nucleoside transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MAS, malate-aspartate shuttle; NAAD, nicotinic acid adenine; NADH, nicotinamide adenine dinucleotide reduced form; NADK, NAD$^+$ kinase; NADP$^+$, nicotinamide adenine; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NAMPT, nicotinamide phosphoribosyltransferase; NMNAT, nicotinamide mononucleotide adenylyltransferase; NNT, nicotinamide nucleotide transhydrogenase; NRK, nicotinamide riboside kinase; OX-PHOS, oxidative phosphorylation; PARP, poly(ADP-ribose) polymerase; PPP, pentose phosphate pathway; SARM1, sterile alpha and TIR motif-containing protein 1; SIRT, sirtuin. Color images are available online.

directly taken up into the cell, it would solely require the NMNAT activity for NAD$^+$ synthesis. However, NRK1 deficiency, inhibition of CD73 (the cell surface protein thought to convert extracellular NMN to NR), and inhibition of ENTs all prevented NAD$^+$ generation from exogenous NMN, providing evidence for conversion of NMN to NR before cellular uptake. The recent discovery of a specific NMN transporter, Slc12a8 (56), will require careful analysis of its cell type expression patterns to resolve this debate.

NAD(H) membrane transport

The transport of NAD(H) across biological membranes has been similarly controversial. The canonical view is that NAD$^+$ is unable to cross lipid bilayers (34). The brush border intestinal lining has been shown to hydrolyze NAD$^+$ to NMN, which is further hydrolyzed to NR and NAM (55). The cell surface protein CD73 has also been shown to catalyze the conversion of NAD$^+$ to NR *via* NMN (48, 57). However, dedicated NAD$^+$ transporters are thought to exist. One way of importing NAD$^+$ is *via* connexin 43 (Cx43) channels, which are permeable to NAD$^+$ (22) and are highly expressed on cardiomyocytes. Indeed, exogenous NAD$^+$ was shown to block cardiac hypertrophy, and this effect was abolished in the presence of the Cx43 blocker carbenoxolone (126). However, despite considerable evidence that exogenous NAD$^+$ (without prior hydrolysis) can be taken up into different types of mammalian cells (17, 127, 167, 175), besides Cx43 the transporters responsible for this are still to be discovered.

Source of mitochondrial NAD$^+$

Whereas cytosolic and nuclear NAD$^+$ pools are considered to be exchangeable *via* diffusion through the nuclear pore, the canonical view is that the mitochondrial NAD$^+$ pool is sequestered due to the impermeability of the inner mitochondrial membrane. Whereas NMNAT3 is found to localize to the mitochondrial matrix, NAMPT is not (111). As such, cytosolic NMN (but not NAM) is widely believed to be the precursor of mitochondrial NAD$^+$, although the exact mechanism of its mitochondrial transport is unknown. Recent evidence suggests that intact NAD$^+$ is able to cross the inner mitochondrial membrane (33, 42, 141), and this was conclusively demonstrated using isotope labeling experiments (33). Whether it is NAD$^+$ or NADH that crosses into mitochondria is still unknown, and the possibility that NMNAT3 still contributes to mitochondrial NAD$^+$ *via* NMN import has not been ruled out. Indeed, experiments using a genetically encoded biosensor have shown that the mitochondrial NAD$^+$ pool is maintained by both direct import of cytosolic NAD$^+$ and synthesis from imported NMN (23). While an NAD$^+$ transporter exists in bacteria (59) and has also been identified in mitochondria of yeast (159) and plants (119), the search for its mammalian counterpart continues.

NAD$^+$-Consuming Pathways and Compartmentalization

Poly(ADP-ribose) polymerases

PARP is a family of enzymes that transfer adenosine diphosphate (ADP)-ribose (ADPR) onto target proteins (Fig. 3).

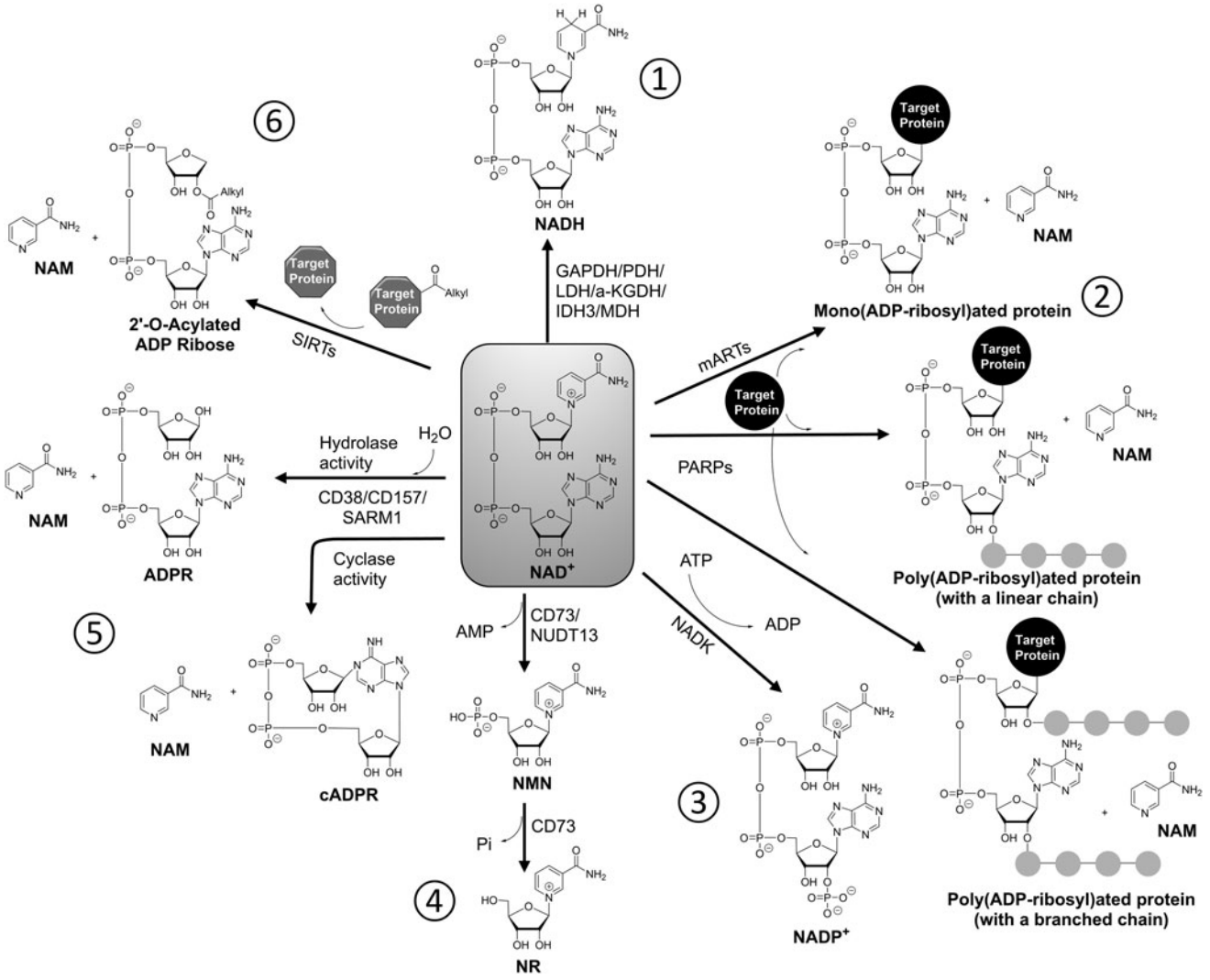

FIG. 3. Cellular fates of NAD+. NAD+ has numerous fates in the cell; *clockwise* from *top*. **(1)** Numerous metabolic enzymes use NAD+ as redox cofactor, with the reduced form (NADH) being a cellular ''reducing equivalent'' used to drive biosynthetic and other reactions. **(2)** mARTs and PARPs catalyze the adduction of ADP-ribosyl groups onto proteins, either singly or in linear or branched chains. This is an important protein post-translational modification that regulates protein function. **(3)** NAD+ can be phosphorylated to make NADP+, which is used as a redox cofactor like NAD+. Its reduced form (NADPH) is used to drive many antioxidant enzymes important for defense against oxidative stress. **(4)** Cell surface CD73 and mitochondrial nudix hydrolase 13 (NUDT13) enzymes have the pyrophosphohydrolase activity, converting NAD+ to NMN. CD73 further converts NMN into NR and plays an important role in cellular uptake of NAD+ and NMN. **(5)** CD38/CD57/SARM1 enzymes can perform either (i) a cyclase activity that generates NAM and cyclic ADPR (cADPR), the latter being an important cell signaling second messenger, or (ii) a hydrolase activity that generates NAM and ADPR. CD hydrolase is considered an NAD+ degradation pathway. **(6)** The SIRT family of lysine deacylases use NAD+ as a substrate, generating NAM while removing an acyl group (*e.g.*, acetyl, succinyl, malonyl) from a protein lysine residue to form 2′-O-acyl-ADPR. ADPR, adenosine diphosphate ribose; mARTs, mono-ADPR transferases.

There are 17 members of the PARP family in humans (69) including both poly-ADPR polymerases (PARP-1, 2, and 5) and mono-ADPR transferases (PARP-3, 4, 6, 7, 10, 12, and 14–16) (90). PARPs are predominantly located in the nucleus (PARPs 1–5, 9, and 14) but are also found in the cytoplasm (PARP-5, 10, 12, 13, and 15), cell membrane (PARP-4, 9, 14, and 16), and ER (PARP-16) (5, 90). Activity (poly *vs.* mono ADP-ribosylation) and location of certain PARP isoforms are still unknown (*e.g.*, PARP-8 and 11).

PARPs bind NAD+ and cleave it into NAM and ADP-ribose. Depending on the activity of the particular PARP isoform, the target protein is either mono or poly(ADP-ribosyl)ated (PARylation). PARylation can either be linear or branched (Fig. 3). PAR polymers can reach lengths of 200 ADPR units (110). Typical amino acid residues of the target protein onto which ADPR is transferred include aspartate, glutamate, asparagine, arginine, lysine, serine, and cysteine (89). The majority of PARP substrates are nuclear proteins involved in DNA synthesis and repair, nucleic acid metabolism, and chromatin structure modulation (104). PARP-1 is also known to PARylate itself (auto-PARylation) (in addition to being PARylated by PARP-2) and is one of the main acceptors of poly-ADPR *in vivo* (80). PARylation is a dynamic process, and PAR polymer is rapidly degraded by poly (ADPR) glycohydrolase (PARG) and ADPR protein lyase (half-life <1 min) (160).

PARPs are thought to be responsible for the majority of NAD^+ consumption both constitutively and upon hyperactivation during DNA damage (140). PARP-1 (85%–90%) and PARP-2 (10%–15%) contribute to most of the total PARP activity (155). The main role of PARPs is to detect DNA damage and initiate DNA repair (38). The extent of PARP activation following DNA damage determines the fate of the cell (117). If the damage is repairable, PARP activation contributes to cell survival by engaging DNA repair machinery. More severe damage engages apoptotic machinery in which caspases cleave PARP-1, thereby inactivating it and preventing the loss of cellular ATP, which is then used to dispose of the cell in a safe manner. In case of extensive DNA damage (*e.g.*, in tissue ischemia and reperfusion injury), PARP hyperactivation causes severe depletion of cellular NAD^+ stores. Salvage of NAD^+ from the resulting NAM is an energy intensive process, which depletes cellular ATP levels. As a result, energy intensive apoptotic machinery is not engaged, and the cell undergoes necrosis. Besides DNA repair, PARPs are involved in regulating inflammation, metabolism, and cell proliferation [reviewed in detail in (5, 94, 104)].

Sirtuins

Sirtuins are a class of NAD^+-dependent proteins with deacylase activity. The term ''sirtuin'' comes from the first member of the family, Sir2 (silent information regulator two), identified in yeast as a protein responsible for transcriptional silencing (137). In mammals, there are seven Sir2 homologs identified as SIRT1–SIRT7. SIRT1, SIRT6, and SIRT7 are predominantly nuclear, SIRT2 is cytosolic, and SIRT3–SIRT5 are mitochondrial (101). Under certain conditions, SIRTs may be present in subcellular compartments different from their primary location. For example, in cardiomyocytes, SIRT1 is located in the nucleus during development but is mostly cytosolic in adult (157). SIRT1 is known to shuttle between the nucleus and the cytosol in response to pathological conditions or stress (70, 171). SIRT3 is also known to localize and function in the nucleus in its full-length form, whereas the processed short form is mitochondrial (73).

The classical reaction catalyzed by sirtuins is the removal of an acetyl group from protein lysine residues, using NAD^+ as an acetyl acceptor. In this process, NAM is released first and the ADPR moiety is covalently attached to the acetyl group on the protein lysine residue. The amide bond between acetyl carbonyl functionality and ε-amino group of lysine is then cleaved, thereby liberating the deacetylated lysine and 2′-O-acetyl-ADP ribose (61). While deacetylation is the main activity of sirtuins, they are also capable of removing other acyl groups in a target protein- and isoform-specific manner. SIRT1–SIRT3 and SIRT7 have the deacetylase activity. SIRT5 has demalonylase and desuccinylase activities in addition to deacetylase (36). SIRT4 has been shown to act as an ADPR transferase, catalyzing the release of NAM, but not the cleavage of the amide bond in the second step of the reaction (60). Like SIRT4, SIRT6 also has the ADPR transferase activity (88) and also removes long-chain fatty acid groups like myristoyl and palmitoyl from lysines (74). Because NAD^+ is necessary for the sirtuin activity, sirtuins can act as a direct link between cellular metabolic status and protein PTMs, in effect acting as ''metabolic sentinels,'' in a manner similar to adenosine monophosphate (AMP)-dependent protein kinase (66). Additionally, NAM, which is a product of the reaction, is a noncompetitive inhibitor of sirtuins.

Sirtuins regulate a plethora of mammalian proteins involved in diverse processes, such as energy metabolism, genomic stability, inflammation, cellular stress resistance, circadian rhythms, tumorigenesis, autophagy, and apoptosis [reviewed in (43, 61)]. Notably, the NAD^+ consumption potential of sirtuins (*i.e.*, specific activity, combined with relative expression levels *vs.* other NAD^+ consumers) is thought to be significantly lower than that of PARPs, as discussed in the Competition for NAD^+ among SIRTs/PARPs/CDs section.

Glycohydrolases

Cluster of differentiation (CD) proteins CD38 and CD157 (Fig. 2) are cell surface glycohydrolases that cleave a glycosidic linkage within NAD^+ to release NAM and ADPR (Fig. 3) (27, 132). ADPR is a product of the hydrolase activity (*i.e.*, it uses water). Both CD38 and CD157 also have the cyclase activity to a smaller extent, in which the NAD^+ molecule is broken down into NAM and cyclic ADPR (cADPR), without using water. The NAM generated in the reactions rapidly enters the salvage pathway to regenerate NAD^+. Both ADPR and cADPR act as second messengers to regulate cytosolic Ca^{2+} flux. This in turn activates various signaling pathways critical for energy metabolism, muscle contraction, immune response, and cell adhesion (58, 121, 132). In addition, recent work has found that CD38 inhibitors can confer protection against cardiac ischemia-reperfusion injury, potentially by enhancing NAD^+ availability for the SIRT activity (21) (see the Balancing NAD Consumption and Redox in a Compartment-Specific Manner section). Besides ADPR and cADPR, CD38 and CD157 also generate nicotinic acid adenine dinucleotide phosphate from $NADP^+$, which also acts as a second messenger for Ca^{2+} mobilization (96).

Sterile alpha and toll-interleukin receptor (TIR) motif-containing 1 (SARM1) protein is the first member of recently discovered class of proteins, which contains two putative protein–protein interaction domains—sterile alpha and TIR—and was thought to play a role in innate immunity in *Caenorhabditis elegans* (31, 103). In recent years, a central role for NAD^+ metabolism in neurodegeneration has emerged, where SARM1 initiates cell destruction by mediating NAD^+ depletion while NMNAT1 protects neurons by maintaining NAD^+ levels (41, 49, 114, 146) [reviewed in (30, 50)]. Interestingly, SARM1's NADase activity is mediated by the TIR domain, which usually is responsible for protein–protein interactions, and is the first example of a TIR domain exhibiting enzymatic activity. SARM1 is triggered by neuronal injury and catalyzes cleavage of NAD^+ into ADPR, cADPR, and NAM (Fig. 3), with NAM acting as a feedback inhibitor of SARM1 (41). Overexpression of NAMPT or NMNAT or supplementation with NR increases NAD^+ synthesis and counteracts axon destruction by SARM1 (49). As such, SARM1 inhibition is emerging as a promising therapeutic strategy in the treatment of acute neuronal injury as well as neurodegeneration and aging.

Pyrophosphohydrolases

CD73 is a cell surface enzyme (Fig. 2) with primary activity as 5′-nucleotidase acting on AMP. It was recently reported to also have the pyrophosphohydrolase activity to

convert NAD^+ to NMN, which is then further converted to NR by its 5′-nucleotidase activity (57). CD73 is required for use of NMN as extracellular NAD^+ precursor (Fig. 3) (153).

Nudix hydrolases are a superfamily of hydrolytic ''housecleaning'' enzymes that catalyze the cleavage of nucleoside diphosphates linked to x (*i.e.*, any moiety) (14). The human genome has 24 nudix hydrolase genes (99), and the enzymes hydrolyze a wide variety of substrates including organic pyrophosphates such as nucleoside di- and triphosphates and dinucleotides, nucleotide sugars, and RNA caps, with varying degrees substrate specificity (99). NUDT13, a member of this superfamily with the pyrophosphohydrolase activity, catalyzes the hydrolysis of NADH to NMNH and AMP, and NADPH to NMNH and 2′,5′-ADP (2). NUDT13 showed marked preference for the reduced form of pyridine nucleotides with its K_M of 0.34 m*M* for NADH and was shown to localize exclusively to mitochondria. Related human NUDT12 protein hydrolyzes NADH and NADPH with 20-fold greater efficiency than NAD^+ and localizes to peroxisomes (1). NUDT12 and NUDT13 are thought to regulate the $NAD(P)^+$/NAD(P)H ratio within peroxisomes, mitochondria, and possibly also in the cytosol (99).

NADH/NAD^+ Metabolic and Redox Roles

Glycolysis

The process by which glucose and other hexoses are lysed to yield the triose pyruvate consists of two halves (Fig. 4). The first or upper part of glycolysis comprises breakdown of glucose into two interchangeable trioses—dihydroxyacetone phosphate and glyceraldehyde-3-phosphate—and consumes two molecules of ATP, the first at hexokinase (65). In the lower half of glycolysis, glyceraldehyde-3-phosphate is converted to pyruvate, yielding two ATPs. The lower reactions occur twice for each glucose, yielding net two ATP per glucose for glycolysis as a whole (145). The first reaction of this lower part is catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the most studied enzymes in all of biology. GAPDH is famously used as a ''housekeeping'' protein or gene in Western blotting or quantitative polymerase chain reaction experiments. GAPDH uses NAD^+ and makes NADH. As such, despite the dogma that phosphofructokinase is the rate-limiting step of glycolysis (98), this requirement for NAD^+ is such that without the oxidized cofactor, glycolysis cannot proceed. Indeed, in the absence of mitochondrial function, pyruvate is converted to lactate by lactate dehydrogenase (LDH), thus recycling NADH to NAD^+ and permitting glycolysis to continue. It could be argued that LDH exists for the sole purpose of recycling NADH. Alternatively, NADH from glycolysis is recycled back to NAD^+ *via* the malate-aspartate shuttle (MAS), transmitting NADH reducing equivalents into mitochondria to join the NADH pool generated by the Krebs' cycle (see the Krebs' Cycle section and Fig. 4). In some metabolically active tissues, cytosolic NADH reducing equivalency can also be shuttled into mitochondria *via* the glycerol-3-phosphate dehydrogenase system, linked to the reduction of coenzyme Q_{10} at the mitochondrial inner membrane (105). Furthermore, it was recently reported that the process of desaturating fatty acids is a significant pathway for recycling of NADH to NAD^+ in the cytosol (78).

Recently, we showed that acute dosing with bolus NAD^+ precursors such as NMN induced a massive and transient cytosolic acidosis, attributable to a burst of glycolytic activity (109). The importance of this phenomenon to the wide-ranging effects of oral NMN supplementation (102) is underappreciated. Furthermore, it should be emphasized that the relative expression levels of GAPDH *versus* other NAD^+-consuming enzymes (SIRTs, PARPs, *etc.*) are such that the bulk of NAD^+ from bolus additions such as this is likely consumed by GAPDH (see the Competition for NAD^+ between redox/metabolic cofactor enzymes *versus* NAD^+ consumers section).

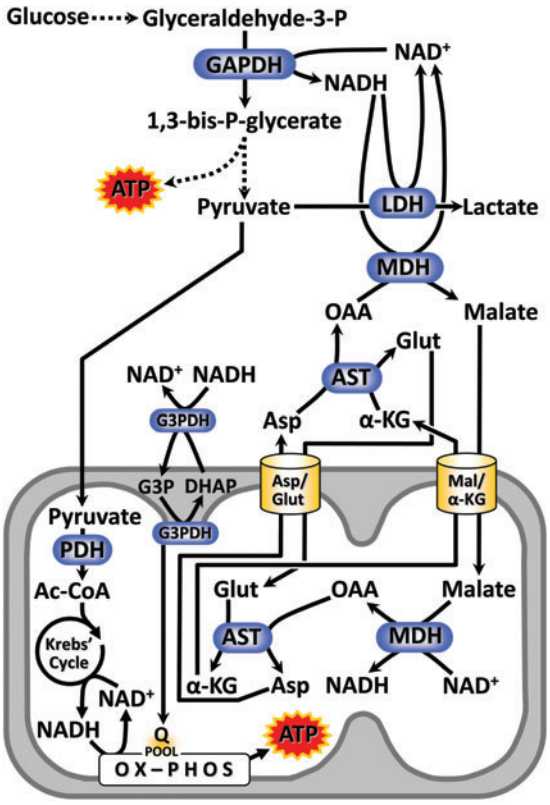

FIG. 4. NADH/NAD^+ redox in metabolism. The enzyme GAPDH, a key step in glycolysis, reduces NAD^+ to NADH. Depending on the functionality of mitochondria, glycolytic NADH has two different routes for reoxidation. In the presence of oxygen (the so called ''aerobic glycolysis''), the reducing equivalency of NADH is transmitted into mitochondria *via* the MAS, comprising cytosolic plus mitochondrial isoforms of MDH working in opposing directions, and cytosolic plus mitochondrial isoforms of AST working in opposing directions. In addition the G3PDH shuttle can transfer NADH reducing equivalents into mitochondria. The mitochondrial import/export of the relevant α-keto acids and amino acids is handled by a pair of electrophoretically driven membrane exchange proteins. The end product of glycolysis, pyruvate, is imported to mitochondria and further oxidized by the Krebs' cycle. In the absence of adequate mitochondrial function (*e.g.*, hypoxia), glycolytic NADH is instead reoxidized by LDH, generating lactic acid as a terminal product (*i.e.*, ''anaerobic glycolysis''). In either scenario, the reoxidation of NADH is an absolute requirement for glycolysis to function. AST, aspartate aminotransferase; G3PDH, glycerol-3-phosphate dehydrogenase. Color images are available online.

Krebs' cycle

The entry of the glycolytic end product pyruvate into the Krebs' cycle in the mitochondrial matrix requires its conversion to acetyl-CoA *via* pyruvate dehydrogenase (PDH), an NADH-generating enzyme (Figs. 4 and 5). The cycle contains three enzymes that reduce NAD^+ to NADH, namely α-ketoglutarate dehydrogenase, malate dehydrogenase (MDH), and IDH3. There are three isoforms of IDH: IDH1 uses $NADP^+$ and resides in the cytosol. IDH2 is mitochondrial and uses $NADP^+$. IDH3 is the NAD^+-dependent mitochondrial isoform that is largely responsible for maintaining Krebs' cycle flux for cellular bioenergetic purposes (145). The mitochondrial $NADP^+$-dependent IDH2 is likely a source of NADPH within the organelle, with the latter required for antioxidant functions (*e.g.*, mitochondrial glutathione reductase [GR]). Further discussion of NADPH-dependent antioxidants is beyond the scope of this review.

While normally the canonical (clockwise) function of the Krebs' cycle results in the generation of NADH, there are a couple of scenarios in which noncanonical functions may exist (Fig. 5): (i) Mutant forms of IDH1 or IDH2 associated with cancer elicit a neomorphic activity that uses NADPH and α-ketoglutarate (α-KG) as substrates to generate $NADP^+$ plus the ''oncometabolite'' D-2-hydroxyglutarate (D-2-HG) (32). In addition, under acidic or hypoxic conditions, either MDH or LDH can adopt a similar neomorphic activity to use NADH to reduce α-KG to the L-enantiomer of 2-HG (72, 108, 113). (ii) Under acidic or hypoxic conditions, mitochondrial IDH2 or IDH3 can run in the reverse direction to convert α-KG back to citrate (108, 169). Such reductive carboxylation of α-KG uses NADH (for IDH3) or NADPH (for IDH2) as reductants and also consumes CO_2 and a proton. This reversal of IDH to eventually generate citrate is a component of cancer metabolism since citrate can be exported from mitochondria and used by ATP-citrate lyase to generate acetyl-CoA, which is then used for lipid biosynthesis (151). By sourcing α-KG from the amino acid glutamine (a preferred fuel for cancer cells), carbon is thus diverted from glutamine to building lipids, an important process for dividing cancer cells. As such, ATP-citrate lyase is an emerging cancer drug target (52).

The noncanonical NADH-consuming reactions of the Krebs' cycle have recently been claimed to be part of a redox sink that exists in hypoxia (92). Specifically, it has been hypothesized that by sinking electrons from NADH into metabolites such as L-2-HG, the cell may avoid ''reductive stress'' (92). However, it should be noted that the bulk flux afforded by such

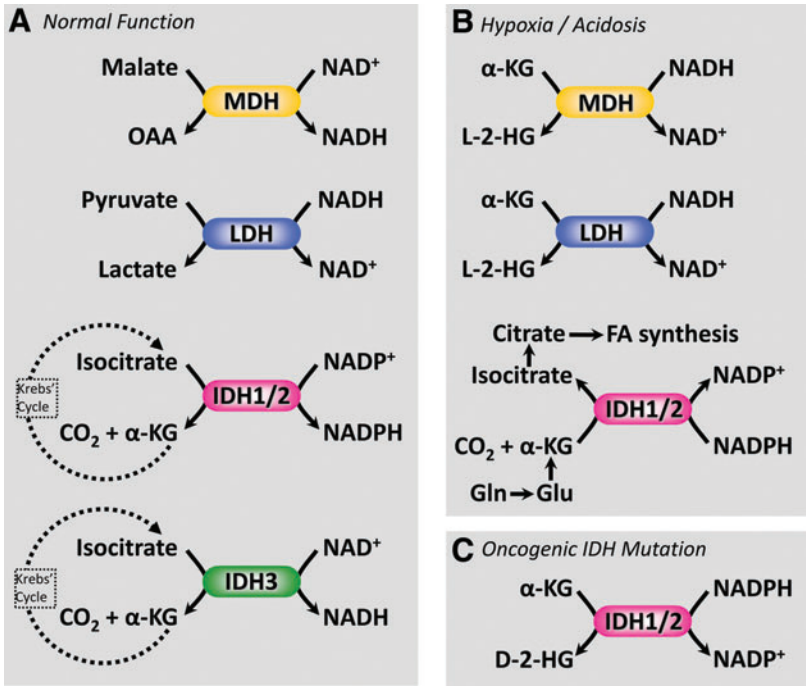

FIG. 5. Noncanonical functions of NAD(P)-dependent dehydrogenases. Several pathophysiological conditions can manifest *neomorphic* enzyme activities from NAD(H)- and NADP(H)-dependent dehydrogenases such as those found in the Krebs' cycle. **(A)** Normal (canonical) function of dehydrogenases: MDH oxidizes malate to OAA, reducing NAD^+ to NADH. LDH reduces pyruvate to lactate, oxidizing NADH to NAD^+. IDH isoforms 1 and 2 decarboxylate isocitrate to α-KG in an $NADP^+$ dependent manner, whereas IDH isoform 3 performs the same reaction in an NAD^+-dependent manner. **(B)** Under conditions of hypoxia and/or acidosis, both MDH and LDH can use NADH to reduce α-KG to the L-enantiomer of 2-hydroxyglutarate (L-2-HG). In addition, IDH 1/2 can operate in reverse, using NADPH to perform reductive carboxylation of α-KG to form isocitrate. The latter is an important biosynthetic pathway in cancer cells, using α-KG derived from the amino acids glutamine or glutamate, to fuel fatty acid synthesis for biomembranes. **(C)** Mutations in IDH 1/2 are associated with aggressive forms of cancer such as glioma and result in a *neomorphic* activity, that is, the ability to use NADPH to reduce α-KG to the D-enantiomer of 2-hydroxyglutarate (D-2-HG). For this reason, D-2-HG is commonly referred to as an ''oncometabolite.'' α-KG, α-ketoglutarate; IDH, isocitrate dehydrogenase; OAA, oxaloacetate. Color images are available online.

pathways is unlikely to be sufficient to impact global redox status. Specifically, for IDH2/MDH/LDH, their rates of conversion of α-KG to L-2-HG are at least three orders of magnitude slower than the canonical activities of these enzymes (108). Furthermore, it has been claimed that L-2-HG and similar metabolic dead-ends may be ''redox reservoirs'' because reverse enzymes exist to return 2-HG to α-KG (92). Again, however, it should be noted that the reverse reactions (catalyzed by L- or D-2-HG dehydrogenases) do not yield NADH (143). Rather, they rely on electron transfer to a flavin adenine dinucleotide (FAD) and then to the ubiquinone cycle embedded in the mitochondrial membrane. Thus, these reactions are not true reservoirs because the product yielded ($FADH_2$) is not the same as the one initially deposited (NADH). Notably, electrons entering the ubiquinone cycle in this manner bypass complex I and thus converting NADH into a 2-HG reservoir and back again sacrifices the proton pumping that would have occurred at complex I.

While these issues do not detract from the importance of 2-HG as a signaling molecule (170, 173), beyond the scope of this review, they highlight that the magnitude and directionality of metabolic fluxes should consider all conditions including relative concentrations and rates for the reactions involved.

Mitochondrial electron transport chain

Complex I of the mitochondrial respiratory chain is the primary consumer of NADH in the organelle, passing electrons *via* a flavin mononucleotide (FMN) and along a ''molecular wire'' comprising at least nine Fe-S centers, eventually onto coenzyme Q_{10} (ubiquinone), and resulting in the pumping of protons from the matrix to the intermembrane space, yielding the energetic intermediate of oxidative phosphorylation (OX-PHOS), the transmembrane proton gradient (145).

Under conditions in which complex I is inhibited (*e.g.*, electron transport chain [ETC] inhibition by lack of oxygen), the NADH/NAD^+ ratio increases, and the convenient autofluorescence of NADH (at 340 nm excitation) is often used as a readout of this phenomenon (147). In this regard, it should be noted that a significant portion of the NADH/NAD^+ pool in mitochondria is thought to be bound to either complex I or the dehydrogenases of the Krebs' cycle, which physically interact with it (20). A study using ''ghost native'' electrophoresis, enabling direct measurements of NADH autofluorescence bound to the complexes, found that the binding of NADH to complex I significantly enhances its fluorescent efficiency (20). As such, measurements of NADH fluorescence are thought to primarily report the bound pool of NADH, not free NADH in the mitochondrial matrix. Thus, it cannot be assumed that changes in NADH fluorescence alone represent meaningful changes in the concentration of free NAD(H) available for use by other enzymes, such as SIRTs. Recent technological advances permitting fluorescent distinction between NADH and NADPH (19) may provide further insight to the interplay of nucleotide pools in mitochondria (18).

Although beyond the scope of this review, it cannot be ignored that the mitochondrial respiratory chain, in particular complexes I and III, is a major source of cellular reactive oxygen species (ROS) (106). Complex I is thought to generate superoxide anion radical ($O_2^{\bullet-}$) from either its matrix-located FMN site or its ubiquinone-binding site. Complex III has two ubiquinone-binding sites, with the outside-facing (Q_O) site (also known as the P-site) being the predominant ROS source. At these sites, the ubisemiquinone radical ($Q^{\bullet}$) can transfer an electron directly onto molecular oxygen to generate $O_2^{\bullet-}$.

In the situation of reperfusion injury following tissue ischemia, a large burst of ROS generation occurs, and the primary source of these ROS is thought to be the reversal of electron transport through complex I, resulting in $O_2^{\bullet-}$ generation at the upstream FMN site (28). Such reverse electron transfer (RET) is thought to be driven by a hyperreduced ubiquinol pool during early reperfusion, which originates from an abundance of succinate, driving ubiquinone reduction by complex II. It is important to state that RET does not necessarily proceed all the way to the NAD(H)-binding site of complex I, and the formation of NADH is not typically observed with RET. Indeed, [NADH] is already very high at the beginning of reperfusion, and this high NADH level may in fact contribute to the diversion of electrons out of the FMN site to form ROS. Regardless the precise mechanisms, the mitochondrion is the proximal source for much of the ROS that the cellular antioxidant machinery has evolved to detoxify.

Cytosolic and mitochondrial NADPH

Although the focus of this review is NADH/NAD^+, the relationship between NAD^+ and its phosphorylated congener $NADP^+$ is highly compartment specific. In the cytosol, NAD^+ kinase (NADK, Fig. 3) phosphorylates NAD^+ to $NADP^+$. NADK was first isolated from *Saccharomyces cerevisiae* by Kornberg and Pricer in 1950 (79) and has since been purified from other organisms. Unlike prokaryotic NADKs, which use inorganic phosphates, human cytosolic NADK requires ATP and Mg^{2+} as cofactors for conversion of NAD^+ to $NADP^+$ (83). NADK is the only pathway for *de novo* $NADP^+$/NADPH biosynthesis. Given the differences in the active site structure and cofactors between prokaryotic and mammalian NADKs, efforts to develop NADK inhibitors as antibacterial agents are underway (15, 120, 124, 150). In recent years, inhibition of human NADK for the treatment of inflammation and cancer has also been proposed (123).

Given the central role NADP(H) plays in regulating crucial processes such as reductive anabolic pathways, signal transduction, and fighting oxidative stress, NADK is critical for normal cellular functions. In the cytosol, the redox state of NADPH is primarily maintained by the initial oxidative stages of the pentose phosphate pathway (PPP) (Fig. 2) (145). NADPH is critical for the maintenance of cellular glutathione redox state (*i.e.*, the balance between reduced [GSH] and oxidized [GSSG], maintained by GR). Indeed, a defect in the first PPP enzyme glucose-6-phosphate dehydrogenase (G6PDH) is one of the most common inherited enzymopathies (26) and results in an inability to maintain glutathione in the reduced form, such that xenobiotics consumption elicits hemolysis.

A mitochondrial isoform of NADK has also been recently reported (112, 179) and was shown to facilitate fatty acid oxidation (FAO), maintenance of mitochondrial SIRT activities, and protection of hepatocytes from oxidative stress (178). In addition, just as in the cytosol, there are numerous

reactions in the mitochondrion that require NADPH, including the re-reduction of GSSG to GSH by mitochondrial GR. As such, specific pathways for the maintenance of NADPH redox status exist in the organelle.

As mentioned above, IDH2 can reduce $NADP^+$ to NADPH (Fig. 5). Alternatively, embedded in the inner mitochondrial membrane, the nicotinamide nucleotide transhydrogenase (NNT) consumes the H^+ gradient to drive the interconversion of NADH+$NADP^+$ to NADPH+NAD^+ (Fig. 2). Notably, the commonly used C57BL/6J mouse strain (from JAX Laboratories) has a polymorphism in the NNT, resulting in an inability to maintain reduced mitochondrial GSH, and an overall increased susceptibility to a wide variety of oxidative stress diseases (139). The C57BL/6N mouse (from EV Taconic) does not have this mutation, making it critical for all mitochondrial investigators to report substrain (J/N) usage.

Recently, it has been proposed that the mitochondrial NNT may exist in concert with ROS-generating dehydrogenases (such as PDH) to form an energy-wasting redox circuit. Specifically, the rate of H_2O_2 production by PDH is a direct function of its flux, and this H_2O_2 is detoxified by the NADPH-consuming GR. The subsequent regeneration of GSH by the NNT consumes the mitochondrial H^+ gradient, thus providing an OX-PHOS uncoupling pathway that is only active at high rates of PDH flux. This overall PDH/NNT complex pathway and its energy-wasting function are thought to be important for whole-organism energetic balance, with implications for diseases such as obesity (45). In addition, it should be noted that NADPH is a critical requirement for the maintenance of other key antioxidants in both the cytosol and mitochondria, namely the thioredoxin, glutaredoxin, and peroxiredoxin systems [see (62) for review].

Transport of NADH reducing equivalents into mitochondria

As mentioned above (the Glycolysis section), glycolysis generates NADH, and this must be converted back to NAD^+ to permit continued glycolytic flux. Under normoxic conditions, this is accomplished by the MAS (Fig. 4). The shuttle consists of a cytosolic isoform of MDH running in reverse to convert oxaloacetate (OAA) to malate, using NADH. Malate is then transported into the mitochondrial matrix, where the Krebs' cycle isoform of MDH converts it back to OAA, regenerating NADH for use by the ETC. To avoid accumulation of OAA in the matrix, OAA is converted to aspartate with the addition of an amino group from glutamate, catalyzed by aspartate aminotransferase (AST), and also yielding α-KG. This α-KG is then transported out of the mitochondrion in exchange for malate coming in. The removal of aspartate and import of glutamate are handled by the mitochondrial glutamate (Glu)/aspartate (Asp) carrier. A corollary isoform of AST in the cytosol then catalyzes the reverse reaction (Asp + α-KG → OAA + Glu). Thus, the MAS can be considered as consisting of two parts—one to carry reducing equivalents of NADH into the mitochondrion and the other to bring the carrier molecules back out again *via* an amino acid shuttle. It should be noted that NADH itself is not transported by the MAS, rather only its reducing equivalency. Also, the enzymes and transporters of the MAS are poised to operate only in one direction under normal conditions. The MAS does not transport NADPH equivalents.

Although the primary function of the MAS is transportation of NADH reducing equivalents for metabolic purposes to facilitate glycolytic flux, the role of this transport system in equilibrating redox power across the membrane (*e.g.*, to provide reducing power for antioxidant systems or to modulate NADH/NAD^+ ratios in the mitochondrion for the purpose of affecting the SIRT activity) is unclear. Furthermore, the existence of the MAS for the purpose of reducing mitochondrial NAD^+ using cytosolic NADH reducing power implies that the import of NAD^+ from the cytosol (see the Source of Mitochondrial NAD^+ section) is likely in the form of NAD^+ and not NADH.

Balancing NAD Consumption and Redox in a Compartment-Specific Manner

Compartment-specific levels of NAD^+ and related metabolites

The estimated total intracellular NAD^+ content in mammalian cells ranges between $\sim$200 and 500 μM (24, 152, 172). However, NAD^+ levels can vary widely in response to diverse stimuli, including glucose deprivation, fasting, caloric restriction, exercise, and circadian cycles (24). In terms of subcellular compartmentation, NAD^+ is typically found in the cytosol and nucleus at $\sim$100 μM and in mitochondria at $\sim$250 μM, although this varies with cell type (23). Generally, the cytosolic pool of NADH/NAD^+ is heavily favored toward the oxidized state, such that NADH/NAD^+ ratio is $\sim$1:1000 under normal conditions (*i.e.*, $\sim$0.1% is in the reduced state) (164). The use of NADH as the major reducing equivalent between the Krebs' cycle and the ETC is such that NADH/NAD^+ ratio in mitochondria is much higher (1:10) compared with cytosol, although the absolute value is highly dynamic depending on respiratory and metabolic activities (164).

In contrast to NAD^+, the cytosolic $NADP^+$ pool is primarily found in the reduced state (*i.e.*, NADPH/$NADP^+$ $\sim$200:1) (164). It should also be noted that in the ER, the redox state is generally more oxidized to facilitate the formation of disulfide bonds for protein folding. Surprisingly, however, indirect measurements have suggested that the ER pool of NAD(P)H is able to remain relatively reduced, despite a prevailing oxidative environment (125).

Even though at the cellular level NADP(H) pools are present in a generally more reduced state than NAD(H), the absolute cellular levels of NADH are reported to be higher than NADPH (53, 174), indicating that the total pool of NAD(H) is bigger than NADP(H).

Competition for NAD^+ among SIRTs/PARPs/CDs

Some studies have suggested that NAD^+-consuming enzymes can compete for NAD^+ within a given cellular compartment. For example, hyperactivation of PARP-1 following ischemia-reperfusion injury in the heart was shown to result in a depression of the SIRT1 activity (7). In addition, PARP-1 and SIRT1 are thought to play reciprocal roles in regulating each other's expression—SIRT1 is able to negatively regulate the PARP-1 promoter and the SIRT1 promoter is also under the influence of PARP-2 (6, 7). Furthermore, SIRT1 is known to deacetylate PARP-1 directly resulting in its inhibition, suggesting that these enzymes may cross talk at both

the genetic and activity levels to coordinate nuclear NAD^+ availability (133).

It has also been shown that NMNAT1 can bind to ADPR polymers that result from the PARP activity. This raises the possibility that NMNAT1 may be recruited to sites of the PARP activity (including PARP auto-PARylation), thus providing more NAD^+ for PARP to function (13). In addition, SIRT1 binds to NMNAT1, helping to recruit NMNAT1 to specific sites, which may help stimulate the SIRT1 activity (180). As such, PARP and SIRT1 may compete not only for NAD^+ itself but also for NMNAT as a source of NAD^+. The complexity of PARP-SIRT interactions is reviewed in Refs. (25, 93).

Similar to PARPs, CD38/CD157 also compete with SIRTs for NAD^+, and these glycohydrolases have been shown to modulate the SIRT activity [reviewed in (97)]. Genetic ablation or pharmacological inhibition of CD38 in mice results in 10- to 30-fold increase in cellular NAD^+ levels (3, 8, 40). These elevated NAD^+ levels serve to activate SIRT1, thereby providing resistance to the development of diet-induced obesity and accompanying glucose intolerance and liver steatosis (8). Similarly, CD38 inhibition has been shown to confer protection against cardiac ischemia-reperfusion injury, potentially by enhancing NAD^+ availability for the SIRT activity (21). The extracellular location of CD38 and its ability to modulate the SIRT activity suggest that cellular SIRT activity can respond dynamically to extracellular NAD^+ availability.

Since many NAD^+-consuming enzymes are inhibited by the by-product NAM, it is likely that enhanced activity of any one class of NAD^+-consuming enzymes (SIRTs, PARPs) may influence the activity of the others not only by reducing the availability of NAD^+ but also by increasing NAM levels.

Competition for NAD^+ between redox/metabolic cofactor enzymes versus *NAD^+ consumers*

A common observation in the cell death field is that PARP-1 hyperactivation leads to an ''energy crisis'' (37). However, this is not necessarily due to depletion of the bioenergetic pool of NAD^+, which is localized in the mitochondrion, while PARP is primarily in the nucleus. Indeed, massive NAD^+ depletion was shown to result in virtually no effect on mitochondrial function for up to 24 h. (128, 154, 172). Instead, the bioenergetic effects of PARP hyperactivation are likely due to the large amounts of ATP required for salvaging NAD^+ from NAM (see the NAD^+ Synthesis and Compartmentation section, Fig. 1). In this regard, an energetic crisis involving NAD^+ depletion is worse than one due to ATP depletion alone. With ATP depletion (*e.g.*, due to mitochondrial dysfunction), glycolysis can continue. However, when NAD^+ depletion is the root cause of ATP depletion, glycolysis is also impaired, leading to a deeper energetic crisis.

While metabolism typically uses NAD^+ as a redox cofactor, SIRTs/PARPs and so on use NAD^+ as a substrate (yielding NAM and ADPR derivatives). A key factor that has received little attention in the competition between these processes is the relative levels of the enzymes concerned. Global analysis of protein expression levels (The Human Protein Atlas) (161) indicates that in most tissues the transcript levels of the key glycolytic enzyme GAPDH are several orders of magnitude greater than NAD^+-consuming enzymes. For example, in human heart, the relative expression levels (in ''TPM'' units—transcripts per million) for *Sirt1*, *Parp1*, and *Gapdh* are 6.9, 32.9, and 2936, respectively. Even in the tissue with the highest levels of *Sirt1* expression (testis), *Sirt1* at 49 TPM is far lower than *Gapdh* at 891 TPM. For the highest *Parp1* expressing tissue (lymph node), *Parp1* at 150 TPM is 10-fold lower than *Gapdh* at 1635 TPM.

Although transcript levels may not be truly indicative of protein levels, in addition to expression, the affinity (K_M) of these enzymes for NAD^+ should also be considered. SIRT1 has a K_M for NAD^+ of $\sim 95\,\mu M$, whereas PARP-1 is ~ 50–$100\,\mu M$ and CD38 is 15–25 μM (24). The K_M for NAD^+ of mammalian GAPDH is $\sim 35\,\mu M$ (122). Given these similar K_M values, it is suggested that competition for NAD^+ between GAPDH and other consuming enzymes (*e.g.*, SIRTs/PARPs) will be based largely on their relative concentrations, such that GAPDH will be the dominant NAD^+ consumer. Indeed, in a recent study, we showed that acute administration of the NAD^+ precursor NMN to cells or intact hearts led to a massive and rapid acidification of the cytosol, concurrent with stimulation of glycolysis (109). Notably, since SIRT1 in the heart is mostly cytosolic, no change in cytosolic lysine acetylation was observed, suggesting no stimulation of the SIRT1 activity by acute NMN.

Metabolism/NAD^+ cross talk at genetic and metabolic levels

In addition NAD^+ being both a metabolic redox cofactor and a substrate to enzymes such as PARPs and SIRTs, it has also been proposed that nuclear NAD^+ regulates the expression of several genes that encode metabolic enzymes. Indeed, a recent study found that compartmentation of NAD^+ synthesis and consumption machineries plays an important role in balancing glucose consumption for energy generation *versus* conversion of glucose into fat for energy storage, *via* regulation of gene transcription (142). Besides its role in DNA repair, PARP-1 regulates the adipogenic transcriptional program (95). The PARylation process converts NAD^+ to NAM, and nuclear NMNAT1 helps to sustain the PARP-1 activity by regenerating NAD^+ from NMN (which itself is generated from NAM by NAMPT, Fig. 2). However, NMNAT2 provides NAD^+ for glycolysis. This competition between NMNAT1 and NMNAT2 for the common substrate NMN helps regulate the balance between energy consumption (glycolysis) and storage (adipocyte differentiation). Thus, NAD^+ serves as a powerful tool to intimately link metabolism and gene regulation.

Another example of the complexity of NADs regulatory role in metabolism and biology is that NADH/NAD^+ and their related metabolites often have allosteric regulatory interactions with the same metabolic enzymes that use the NADH/NAD^+ redox couple as a cofactor. One example is mitochondrial Cx-I (NADH ubiquinone oxidoreductase), which is allosterically activated by ADPR (54). Another example is that NADH allosterically activates PDH kinase, which phosphorylates PDH resulting in its inhibition (145). As such, the accumulation of the product of the Krebs' cycle NADH serves to slowdown the entry of carbon into the cycle in the form of acetyl-CoA.

Furthermore, it should not be forgotten that the acetyl groups that are used to modify lysine residues (*i.e.*, the same

acetyl groups that NAD^+-dependent SIRTs remove) originate from acetyl-CoA. Although lysine acetyltransferase enzymes exist (148), a significant portion of cellular acetylation may derive from nonenzymatic reactions of acetyl-CoA at slightly alkaline pH (such as found in the mitochondrion) (165). Thus, lysine acetylation can be viewed as reflecting the overall acetyl-CoA/NAD^+ balance within a given cell compartment. Generally, deacetylation of metabolic enzymes involved in FAO leads to their stimulation (67). As such, under conditions of high workload in which respiratory complex I generates more NAD^+, SIRT-mediated deacetylation may stimulate FAO to provide more ATP for the higher workload. Conversely, under conditions of low workload, the accumulation of acetyl-CoA (due to feedback inhibition of the Krebs' cycle) leads to acetylation and inhibition of FAO proteins. This is illustrated in Figure 6.

On a related note, it is reported that complex I defects can lead to the inhibition of mitochondrial sirtuins (75). Specifically, the *Ndufs4*$^{-/-}$ mouse model of Cx-I deficiency exhibits hyperacetylation of mitochondrial proteins. This has been attributed to the inhibition of SIRT3 activity due to an increased NADH/NAD^+ ratio. However, although SIRTs can be inhibited by NADH (87), this only occurs at very high NADH/NAD^+ ratios that are not relevant *in vivo*. At physiological NADH/NAD^+ ratios (1:10, see the Compartment-specific levels of NAD^+ and related metabolites section), SIRTs are unaffected by NADH. Notably, in the *Ndufs4*$^{-/-}$ mouse, the driver of the higher NADH/NAD^+ ratio was an increase in NADH, whereas NAD^+ levels remained static. A more likely explanation for the hyperacetylation is that Cx-I inhibition and NADH buildup triggered feedback inhibition of the Krebs' cycle, leading to the accumulation of acetyl-CoA, which drives lysine acetylation. Clearly, when using surrogate markers such as global lysine acetylation to infer the SIRT activity, the acetylation side of the equation should not be overlooked.

An important caveat in the field of lysine acylation PTMs is the apparent discord between phenotypic effects and the stoichiometry of acylation (*i.e.*, site occupancy on a given residue). Although reports of acetylation stoichiometry are generally low ($\sim$1%), an emerging concept is that overall ''lysine load'' from various PTMs (acetylation, sumoylation, ubiquitination, succinylation, glutarylation, *etc.*) may be much higher (4, 100).

Further evidence for the intricate cross talk between sirtuin activity, redox state, and metabolic regulation is seen with the proposed role of SIRT5 as a regulator of two important NADPH-generating enzymes, namely IDH2 and G6PDH. Activation of SIRT5 by oxidative stress (H_2O_2) is thought to trigger the deacylation of these enzymes, activating them to generate NADPH to drive an antioxidant defense (181). In a similar manner, in addition to its role as a regulator of mitochondrial bioenergetics [*e.g.*, deacetylation of ATP synthase (163)], SIRT3 is also reported to deacetylate and activate mitochondrial MnSOD as part of an antioxidant response to metabolic perturbation (35).

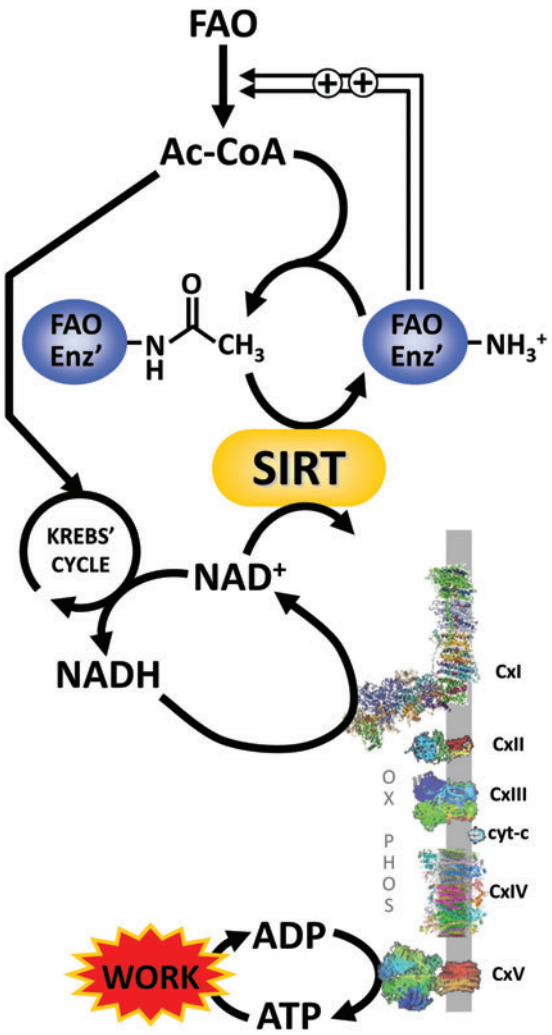

FIG. 6. Lysine acetylation as a metabolic signal in the regulation of fatty acid β-oxidation. It is generally recognized that lysine acetylation of FAO enzymes (FAO Enz′) is inhibitory. In tissues such as the heart that rely heavily on FAO for bioenergetic needs, the ratio of acetyl-CoA (Ac-CoA) to NAD^+ can serve as a metabolic signal for workload, to adjust FAO accordingly to provide ATP. Under conditions of low work, Krebs' cycle activity is low, leading to build up of Ac-CoA, which results in acetylation and inhibition of FAO enzymes. This may be an important feedback mechanism to prevent acyl-carbon-stress due to too much accumulation of acyl-CoA groups. Contrastingly, under conditions of high workload NADH will be rapidly oxidized by respiratory complex I (CxI) of OX-PHOS, yielding NAD^+, which stimulates sirtuin activity to deacetylate (and thus activate) FAO enzymes. Resulting stimulation of FAO provides the ATP needed to drive the increased workload. FAO, fatty acid oxidation. Color images are available online.

Conclusions and Therapeutic Considerations

In summary, there are numerous levels at which the enzymes that use NAD^+ as a substrate, and those that use the NADH/NAD^+ redox couple as a cofactor, compete for this essential nucleotide. While early studies in this area made bulk assumptions regarding the interchangeability of the cellular NADH/NAD^+ pools, recent discoveries about the subcellular localization of these enzymes has led to a more nuanced understanding, and the notion of isolated NADH/NAD^+ pools in specific cell compartments. As such, it cannot be assumed that these pools are interchangeable.

While a thorough review of the various pharmacological/therapeutic approaches to manipulation of NAD^+ and its consuming enzymes is beyond the scope of this review [reviewed in (134)], the compartmentation and pleiotropic roles of NAD^+ in the cell outlined herein suggest that the manipulation of NAD^+ and its consuming enzymes *en masse* may carry unforeseen risks, due to impacts on other parts of the NAD^+ cellular network. This is important because NAD^+ supplementation is now receiving considerable attention in the field of aging.

NAD^+ levels are known to decline with age (51), and indeed, the sirtuin family of NAD^+-dependent proteins was initially discovered within the context of the anti-aging effects of caloric restriction. Several anti-aging therapeutic approaches including caloric restriction are thought to involve in the effects on NAD^+ homeostasis (51). However, the complexities of NAD^+ homeostasis should be appreciated during the design of therapeutic interventions for *in vivo* manipulation of NAD^+. This is perhaps best illustrated by discussing one of the primary targets of such interventions—SIRT1.

SIRT1 has been shown to play a key role in the regulation of autoimmunity. Specifically, the SIRT1 activity is involved both in the suppression of regulatory T cells, which protect against autoimmunity (82, 162), and in the activation of T helper 17 CD4 cells, which contribute to autoimmunity (86). As such, global SIRT1 activation might confer an increased risk of autoimmune diseases.

In cancer, evidence exists for both tumor prevention (44) and promotion by SIRT1 (84). In addition, SIRT3 has been shown to function as a tumor suppressor (77). Notably, some tumors show increased NAMPT expression, such that interventions that increase NAD^+ might enhance or promote tumor development (149). Similarly, given the importance of glycolysis for cancer cell metabolism (the Warburg effect), our results showing that acute supplementation of NMN results in bulk stimulation of glycolysis (109) suggest that the use of such compounds to boost NAD^+ availability should be considered carefully, to avoid a pro-cancer metabolic environment.

Both SIRT1 and SIRT2 play a critical role in regulating neurodegeneration but act in opposite directions (116, 168). SIRT1 overexpression has been shown to be beneficial for neuronal survival in tissue culture and animal models of Alzheimer's disease, amyotrophic lateral sclerosis, and polyglutamine toxicity (76, 85). However, SIRT2 has been implicated in neuronal death, and genetic and pharmacological inhibition of SIRT2 has been shown to rescue α-synuclein toxicity models of Parkinson's disease (115). These observations underline the importance of tailoring therapeutic interventions that specifically stimulate NAD^+ levels in the desired subcellular compartment (in this case, the nucleus) *versus* stimulating global NAD^+ levels to avoid undesired side effects.

While the above examples highlight the importance of stimulating NAD^+ levels in a target cell type- and subcellular compartment-specific manner, to stimulate only the desired enzyme isoform, in some instances doing so might not be enough. For example, in the liver, SIRT1 regulates gluconeogenesis and fat homeostasis by deacetylating various proteins involved in these processes, but these deacetylation events can have opposing effects. SIRT1 deacetylation of PGC-1α activates gluconeogenesis (138), whereas deacetylation of CRTC2, another coactivator of gluconeogenesis, triggers its ubiquitination and degradation (91).

SIRT1 function also changes depending on the pathological state in certain tissues. SIRT1 has been shown to protect kidney and suppress diabetic albuminuria (64). On the contrary, SIRT1 is implicated in the pathophysiology of autosomal-dominant polycystic kidney disease (ADPKD) (182). ADPKD is marked by the development of multiple bilateral cysts that replace normal kidney tissue, and genetic or pharmacological ablation of the SIRT1 activity delays cyst growth in animal model of this disease. As such, SIRT1 activation is beneficial under normal conditions, whereas its inhibition is beneficial in the disease state.

NAD^+ precursor supplementation for the purpose of increasing cellular NAD^+ levels is already undergoing clinical trials (NCT NCT03151239, NCT03432871, NCT03423342, NCT03501433, NCT02835664). However, large scale trials of nicotinic acid (NA, niacin) for cardiovascular disease (NCT00461630 and NCT00880178) showed efficacy but with considerably more adverse side effects. As such, the aforementioned complex roles of NAD^+ in disease biology suggest that simple nutraceutical approaches to alter NAD^+ levels may not represent a clinical panacea.

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Address correspondence to:
Dr. Paul S. Brookes
Department of Anesthesiology
University of Rochester Medical Center
Box 604, 601 Elmwood Avenue
Rochester, NY 14642

E-mail: paul_brookes@urmc.rochester.edu

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Abbreviations Used

α-KG = α-ketoglutarate
2-HG = 2-hydroxyglutarate
3-HAA = 3-hydroxyanthranilic acid
ACMS = aminocarboxymuconate semialdedhye
ACMSD = aminocarboxymuconate semialdehyde decarboxylase
ADP = adenosine diphosphate
ADPKD = autosomal-dominant polycystic kidney disease
ADPR = adenosine diphosphate-ribose
AMP = adenosine monophosphate
AMS = 2-aminomuconate semialdehyde
Asp = aspartate
AST = aspartate aminotransferase
ATP = adenosine triphosphate
Cx43 = connexin 43
ENT = equilibrative nucleoside transporter
ER = endoplasmic reticulum
ETC = electron transport chain
FAD = flavin adenine dinucleotide
FAO = fatty acid oxidation
FMN = flavin mononucleotide
G6PDH = glucose-6-phosphate dehydrogenase
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
Glu = glutamate
GR = glutathione reductase
GSH = glutathione reduced form
GSSG = glutathione oxidized form
IDH = isocitrate dehydrogenase
LDH = lactate dehydrogenase
MAS = malate-aspartate shuttle
MDH = malate dehydrogenase
NA = nicotinic acid
NAAD = nicotinic acid adenine dinucleotide
NAD^+ = nicotinamide adenine dinucleotide
NADH = nicotinamide adenine dinucleotide reduced form
NADK = NAD^+ kinase
$NADP^+$ = nicotinamide adenine dinucleotide phosphate
NADPH = nicotinamide adenine dinucleotide phosphate reduced form
NAM = nicotinamide
NAMPT = nicotinamide phosphoribosyltransferase
NAR = nicotinic acid riboside
NAMN = nicotinic acid mononucleotide
NMN = nicotinamide mononucleotide
NMNAT = nicotinamide mononucleotide adenylyltransferase
NNT = nicotinamide nucleotide transhydrogenase
NR = nicotinamide riboside
NRK = nicotinamide riboside kinase
OAA = oxaloacetate
OX-PHOS = oxidative phosphorylation
PARP = poly(ADP-ribose) polymerase
PDH = pyruvate dehydrogenase
PNP = purine nucleoside phosphorylase
PPP = pentose phosphate pathway
PRPP = phosphoribosyl pyrophosphate
PTMs = post-translational modifications
RET = reverse electron transfer
ROS = reactive oxygen species
SARM1 = sterile alpha and TIR motif-containing protein 1
SIRT = sirtuin
TIR = toll-interleukin receptor
TPM = transcripts per million